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Pyruvate Kinase of *Streptococcus lactis*

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The kinetic properties of pyruvate kinase (ATP:pyruvate-phosphotransferase, EC 2.7.1.40) from *Streptococcus lactis* have been investigated. Positive homotropic kinetics were observed with phosphoenolpyruvate and adenosine 5'-diphosphate, resulting in a sigmoid relationship between reaction velocity and substrate concentrations. This relationship was abolished with an excess of the heterotropic effector fructose-1,6-diphosphate, giving a typical Michaelis-Menten relationship. Increasing the concentration of fructose-1,6-diphosphate increased the apparent V_{max} values and decreased the K_m values for both substrates. Catalysis by pyruvate kinase proceeded optimally at pH 6.9 to 7.5 and was markedly inhibited by inorganic phosphate and sulfate ions. Under certain conditions adenosine 5'-triphosphate also caused inhibition. The K_m values for phosphoenolpyruvate and adenosine 5'-diphosphate in the presence of 2 mM fructose-1,6-diphosphate were 0.17 mM and 1 mM, respectively. The concentration of fructose-1,6-diphosphate giving one-half maximal velocity with 2 mM phosphoenolpyruvate and 5 mM adenosine 5'-diphosphate was 0.07 mM. The intracellular concentrations of these metabolites (0.8 mM phosphoenolpyruvate, 2.4 mM adenosine 5'-diphosphate, and 18 mM fructose-1,6-diphosphate) suggest that the pyruvate kinase in *S. lactis* approaches maximal activity in exponentially growing cells. The role of pyruvate kinase in the regulation of the glycolytic pathway in lactic streptococci is discussed.

During preliminary studies in this laboratory on carbohydrate metabolism by lactic streptococci (*Streptococcus lactis* and *Streptococcus cremoris*) the complete sequence of glycolytic enzymes was found, except for the apparent absence of pyruvate kinase. Pyruvate kinase also appeared to be absent from extracts of *Bacillus subtilis* (3). However, it was found that the enzyme from *B. subtilis* was labile and required the presence of phosphoenolpyruvate (PEP), KCl, and high protein concentrations for stability. Pyruvate kinases from other organisms have been found to be markedly activated by the intermediary metabolites fructose-1,6-diphosphate (FDP) and adenosine 5'-monophosphate (AMP) (5, 7, 13, 14, 16, 21).

The present investigation was undertaken to determine the factors affecting the activity of pyruvate kinase from lactic streptococci. It was found that the activity of pyruvate kinase from *S. lactis* was enhanced by FDP at the PEP concentration present in growing cells, and it is suggested that this may provide an important mechanism for the regulation of glycolysis in lactic streptococci.

MATERIALS AND METHODS

Organisms and culture conditions. *S. lactis* strains ML_3 and C_{10} and *S. cremoris* AM_2 were grown at 32 C in T5 medium as described previously (19). In this medium *S. lactis* ML_3 grows exponentially (doubling time of 32 min) to a density of 1 mg (dry weight) of bacteria per ml. All results in this paper refer to *S. lactis* ML_3 unless stated otherwise. Bacterial mass was determined as described previously (18).

Pyruvate kinase assay. Assays were carried out at 25 C by following spectrophotometrically the oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm. Unless otherwise specified the standard reaction mixture consisted of: 50 mM triethanolamine-hydrochloride buffer (pH 7.5), 8 mM $MgCl_2$, 80 mM KCl, 0.12 mM NADH, 2 mM PEP, 5 mM adenosine 5'-diphosphate (ADP), 2 mM FDP, 100 μg of lactate dehydrogenase (LDH), and enzyme sample in a final volume of 2.5 ml. PEP, ADP and FDP were dissolved in triethanolamine-hydrochloride buffer and the pH adjusted to 7.5; NADH was dissolved in 1 mM NaOH. Assays using crude extracts were initiated by the addition of PEP, and all other assays were initiated by the addition of 5 to 25 μliters of pyruvate kinase solution. Reaction rates were corrected for NADH oxidase activity and were proportional to enzyme concentration. One unit (U) of pyruvate kinase activity is defined as the amount of enzyme which utilized 1 μmol of PEP per min under the assay conditions described, assuming that the ratio of NADH oxidized to PEP utilized is unity.

Both K^+ and Mg^{2+} were required for pyruvate kinase activity.

Intracellular concentration of metabolites. Cells from exponentially growing cultures (0.4 mg [dry

weight]/ml) were collected from 25 ml of medium on a 47-mm diameter membrane filter (Millipore Corp., 0.8-μm pore diameter), and the intracellular metabolites were extracted by two different procedures. In the first method (9), the filter was placed on a stainless-steel block which was partially immersed in liquid N_2. The filter was then placed in 5 ml of 0.6 N $HClO_4$ at 0 C, and the cells were dispersed by thorough mixing. In the second method (15), the filter was placed in 5 ml of 10% trichloroacetic acid for 30 min. The total time between the commencement of filtration and either the freezing of the filter or its immersion in trichloroacetic acid was 10 to 15 s.

Extracts were assayed for PEP, ADP, and FDP by fluorometric enzymatic analysis at 25 C with NADH indicator systems (9). An excitation wavelength of 350 nm and an emission wavelength of 450 nm were used, and fluorescence blanks were performed on all reagents.

PEP and ADP were measured by coupling pyruvate kinase and LDH reactions and measuring the decrease in fluorescence of NADH after completion of the reaction. The reaction mixture contained: 50 mM imidazole-hydrochloride buffer (pH 7.0), 4 mM $MgCl_2$, 80 mM KCl, 5 μM NADH, 20 μg of LDH, 8 μg of pyruvate kinase, either 200 μM ADP or 80 μM PEP (for PEP and ADP assays, respectively), and the extract sample in a final volume of 2.5 ml. The presence of pyruvate in extract samples was corrected for by omitting pyruvate kinase from the reaction mixture.

FDP was measured by coupling aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase and measuring the decrease in fluorescence of NADH after completion of the reaction. The reaction mixture contained 50 mM imidazole-hydrochloride buffer (pH 7.0), 5 μM NADH, 20 μg of aldolase, 4 μg of triosephosphate isomerase-glycerophosphate dehydrogenase mixture, and extract sample in a final volume of 2.5 ml. A correction was made for the presence of triosephosphates in extract samples by omitting aldolase from the reaction mixture.

Determination of intracellular volume. The intracellular volume of organisms harvested from cultures in the exponential growth phase was determined by the method of Black and Gerhardt (2). Using this thick cell suspension technique the volume outside the plasma membrane was determined with [U-^{14}C]sucrose. The total available fluid space was determined with tritiated water and also by measurement of water loss after drying the packed cell pellet.

Protein assay. Protein determinations were carried out by a modification (1) of the method of Lowry et al. (10).

Purification of S. lactis pyruvate kinase. All steps were carried out at 0 to 5 C.

Step 1: preparation of cell-free extract. Organisms were harvested by centrifugation from exponentially growing cultures at a cell density of 0.3 mg (dry weight)/ml. The deposited bacteria were washed, suspended at a cell density of 10 mg (dry weight)/ml in 0.01 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) containing 0.01 M $MgCl_2$, and disrupted by being shaken with glass beads (19). The cell-free extract was obtained after filtration and centrifugation (35,000 $\times$ g, 20 min).

Step 2: streptomycin sulfate treatment. After the addition of ethylenediaminetetraacetic acid (0.01 mM final concentration), streptomycin sulfate (10%, wt/vol) was added dropwise to the stirred cell-free extract until no further precipitation occurred. The precipitate was removed by centrifugation. Measurement of absorbancy at 260 and 280 nm indicated that this treatment removed more than 95% of the nucleic acids, whereas most of the pyruvate kinase activity was retained.

Step 3: ammonium sulfate precipitation. Finely ground $(NH_4)_2SO_4$ (7.6 g) was slowly added to 20 ml of the cell-free extract with constant stirring. After 2 h the resulting precipitate was removed by centrifugation and discarded. A further 2.7 g of $(NH_4)_2SO_4$ was then added to the supernatant fluid. After 4 h the resulting precipitate was sedimented by centrifugation and dissolved at a concentration of 3 to 5 mg of protein per ml in 0.01 M phosphate buffer (pH 6.5) containing 0.01 M $MgCl_2$ and 3 mM β-mercaptoethanol.

Step 4: gel filtration. A 0.5-ml sample was then placed on a Sepharose 6B column (1 by 25 cm), equilibrated with 0.01 M phosphate buffer (pH 6.5) containing 0.01 M $MgCl_2$ and 3 mM β-mercaptoethanol, and eluted with the same buffer. Fractions (0.5 ml) were collected, and from each a sample (0.1 ml) was removed for protein assay. Glycerol (0.4 ml) was immediately added to the remaining fraction, and the solution was mixed. Fractions were stored at -70 C.

Chemicals. The enzymes LDH and pyruvate kinase were obtained as glycerol solutions from Boehringer, Mannheim, Germany. PEP, FDP, adenosine phosphates, NADH, aldolase, and α-glycerophosphate dehydrogenase-triosephosphate isomerase were obtained from Sigma Chemical Co., St. Louis, Mo. Radioisotopes were obtained from The Radiochemical Centre, Amersham, England. Biochemical grade $(NH_4)_2SO_4$ was obtained from Merck, Darmstadt, Germany, and low-fluorescence blank imidazole was obtained from Sigma.

RESULTS

Purification of pyruvate kinase. Pyruvate kinase from *S. lactis* precipitated between 63 and 80% $(NH_4)_2SO_4$ saturation. The enzyme from *B. subtilis* (3) and *Bacillus licheniformis* (20) precipitated at similar $(NH_4)_2SO_4$ concentrations, whereas the enzyme from *Escherichia coli* precipitated at a lower $(NH_4)_2SO_4$ concentration (13). Pyruvate kinase from *S. lactis* was eluted from a Sepharose 6B column as a single symmetrical peak. Prior to gel filtration, pyruvate kinase was stable with no loss in activity occurring on storage for 5 days at 3 C. After gel filtration the enzyme was unstable in buffer, losing 50% of its activity in 12 h at 3 C. However, the addition of glycerol stabilized the enzyme so that no decrease in activity was observed on storage for 10 days at 3 C. No evidence was obtained for loss in activity during assay of the crude or purified enzyme. The

concentration of inorganic phosphate (P_i) introduced into the enzyme assay system by the pyruvate kinase extract was less than 0.05 mM. At this concentration, phosphate inhibition was negligible (Fig. 1).

The specific activity of the crude enzyme fraction (3.0 U/mg of protein) was increased by $(NH_4)_2SO_4$ fractionation (24 U/mg of protein) and gel filtration (74 U/mg of protein). Thus the overall purification of the enzyme was approximately 25-fold. No detectable NADH oxidase activity was found in the purified enzyme preparation.

Effect of pH on pyruvate kinase activity. The activity of *S. lactis* pyruvate kinase in the presence of 2 mM PEP, 5 mM ADP, and 2 mM FDP was measured in the pH range 5.6 to 8.0 and was found to progress optimally at pH 6.9 to 7.5 in either 0.05 M imidazole-hydrochloride, 0.05 M cacodylate-hydrochloride, or 0.05 M triethanolamine-hydrochloride buffers. This broad pH optimum is similar to that reported for pyruvate kinase from *B. licheniformis* (21) and *E. coli* (13).

Inhibition of pyruvate kinase by ammonium sulfate and P_i. Both $(NH_4)_2SO_4$ and P_i inhibited *S. lactis* pyruvate kinase in the presence of 2 mM PEP, 5 mM ADP, and 2 mM FDP. Potassium sulfate at a concentration of 21 mM caused 50% inhibition of enzyme activity (Fig. 1). Ammonium chloride (40 mM) caused no inhibition, indicating that $(NH_4)_2SO_4$ inhibition was due to SO_4^{2-}. Therefore, LDH in glycerol solution was used for enzyme assays since $(NH_4)_2SO_4$ suspensions of LDH introduced inhibitory levels of SO_4^{2-} into the reaction mixture. Inorganic phosphate (K_2HPO_4-KH_2PO_4, pH 7.4) was a potent inhibitor of pyruvate kinase, causing 50% inhibition at 1.3 mM concentration (Fig. 1). Phosphate and SO_4^{2-} did not inhibit the LDH reaction in assay systems.

Activation of pyruvate kinase by FDP, PEP and ADP. The saturation curve for PEP in the presence of 5 mM ADP was sigmoidal (Fig. 2a). PEP showed homotropic cooperativity (Hill coefficient = 3.3, Fig. 2b) in the absence of FDP and had an approximate Michaelis constant (K_m) of 4 mM. FDP transformed the sigmoidal saturation curve of PEP to a hyperbolic curvature (Fig. 2a) and changed the Hill coeffi-

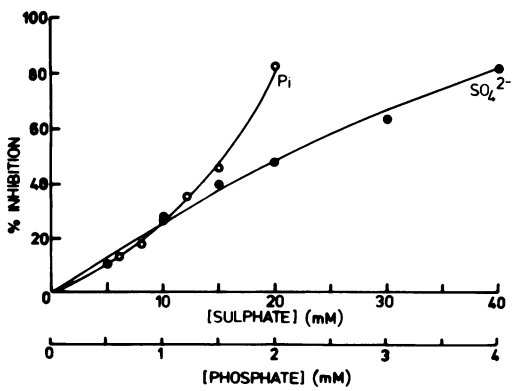

FIG. 1. *Inhibition of S. lactis pyruvate kinase by SO_4^{2-} (●) and P_i (○). The standard assay was used except that the inhibitor was present at concentrations indicated on the graph. The reaction was initiated by adding 0.011 U of pyruvate kinase per ml (0.15 μg of protein per ml).*

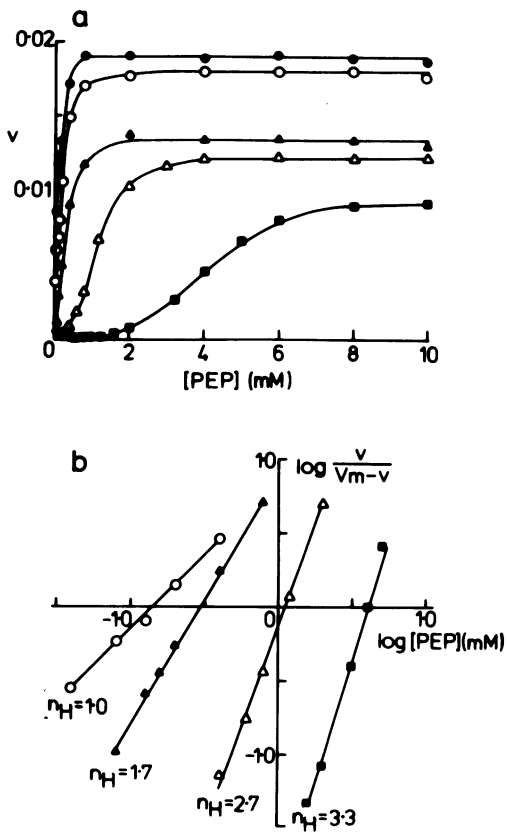

FIG. 2. *Relationship between reaction velocity (v, units per milliliter) of S. lactis pyruvate kinase and PEP concentration with varying concentrations of FDP. The standard assay was used except that the PEP concentration was varied as indicated, and FDP was present at the following concentrations: no addition, ■; 0.08 mM, △; 0.2 mM, ▲; 0.8 mM, ○; 2 mM, ●. The reaction was initiated by adding 0.019 U of pyruvate kinase per ml (0.22 μg of protein per ml). (a) Saturation curves for PEP. (b) Hill plot. The velocities at 10 mM PEP were taken as the maximal velocities (V_m).*

cient from 3.3 to unity (Fig. 2b). In the presence of saturating FDP (0.8 mM), the apparent K_m for PEP of the fully activated enzyme was only 0.14 mM (Fig. 2b). The FDP effect on the saturation curves for ADP was similar, although weaker. In the presence of 2 mM PEP, saturation curves were sigmoidal at low FDP concentrations but changed to a hyperbolic shape at high FDP concentrations (Fig. 3). The saturation curves for PEP and ADP (Fig. 2a and 3a, respectively) show that the apparent maximal velocity (V_{max}) increases with increasing FDP concentrations, and Hill plots of these data show the marked decrease in apparent K_m values with increasing concentrations of FDP (Fig. 2b and 3b). In the presence of 2 mM PEP and 5 mM ADP, the FDP concentration giving one-half maximal velocity was 0.07 mM. Decreasing the concentration of PEP or ADP caused an increase in this value (data not shown). A similar FDP activation was found for the pyruvate kinase from *S. lactis* C_{10} and *S. cremoris* AM_2. Control experiments verified that this effect was due to direct activation of pyruvate kinase and not the auxiliary LDH enzyme.

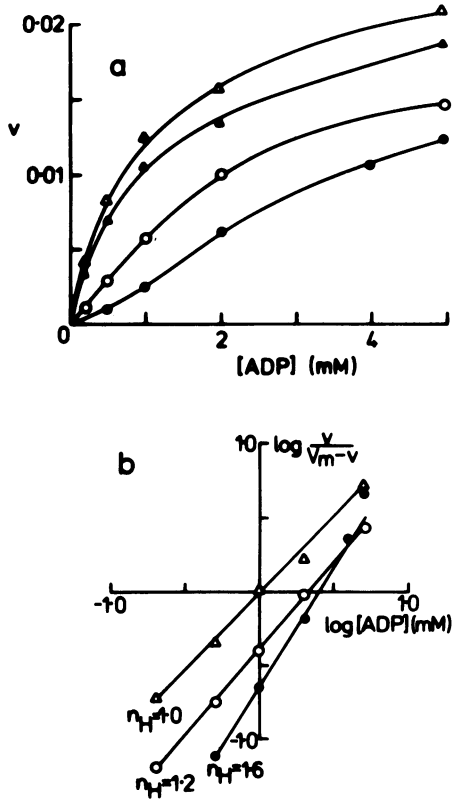

FIG. 3. *Relationship between reaction velocity (v, units per milliliter) of S. lactis pyruvate kinase and ADP concentration with varying concentrations of FDP. The standard assay was used except that the ADP concentration was varied as indicated, and FDP was present at the following concentrations: 0.08 mM, ●; 0.2 mM, ○; 0.8 mM, ▲; 2 mM, △. The reaction was initiated by adding 0.021 U of pyruvate kinase per ml (0.29 μg of protein per ml). (a) Saturation curve for ADP. (b) Hill plot. The V_m values were obtained from a double-reciprocal plot.*

The effect of PEP and ADP on the reaction rate of pyruvate kinase was measured with five concentrations of each substrate in the presence of 2 mM FDP. Results are presented in Fig. 4a and 5a as double-reciprocal plots of reaction velocity (v) versus substrate concentration (S). The K_m for PEP and ADP and the V_{max} for the pyruvate kinase reaction were derived from double-reciprocal plots of the apparent V_{max} (reciprocal of intercept on $1/v$ axis) against substrate concentrations (Fig. 4b and 5b). The K_m values (negative reciprocal of intercept on $1/S$ axis) for PEP and ADP were 0.17 and 1 mM, respectively, and the V_{max} (reciprocal of intercept on $1/V_{max}$ apparent axis) for the reaction was 98 U/mg of protein.

Effect of ATP and AMP. In the presence of 2 mM PEP, 5 mM ADP, and 2 mM FDP, addition of either adenosine 5'-triphosphate (ATP) or AMP at concentrations up to 5 mM had no effect on the reaction rate of pyruvate kinase. However, when the FDP concentration was reduced 10-fold, 5 mM ATP caused 50% inhibition.

Intracellular concentrations of PEP, ADP and FDP. Both extraction procedures released similar concentrations of PEP, ADP, and FDP from growing organisms. Further disruption of cells by shaking with glass beads did not release more of these intermediates, indicating that complete extraction was achieved. Determination of the intracellular fluid volume of *S. lactis* ML_3 indicated that 1 g (dry weight) of bacteria had a protoplast volume of 1.6 ml (J. Thompson, unpublished data). This volume was used to calculate the intracellular concentration of PEP, ADP, and FDP (Table 1). The concentration of FDP in exponentially growing cells was 250 times that required for one-half maximal velocity of pyruvate kinase in vitro with 2 mM PEP and 5 mM ADP, whereas the intracellular concentrations of PEP and ADP were significantly greater than the concentrations required for one-half maximal velocity in the presence of 2 mM FDP (Table 1).

DISCUSSION

The activity of pyruvate kinase from *S. lactis* was dependent upon several factors in vitro.

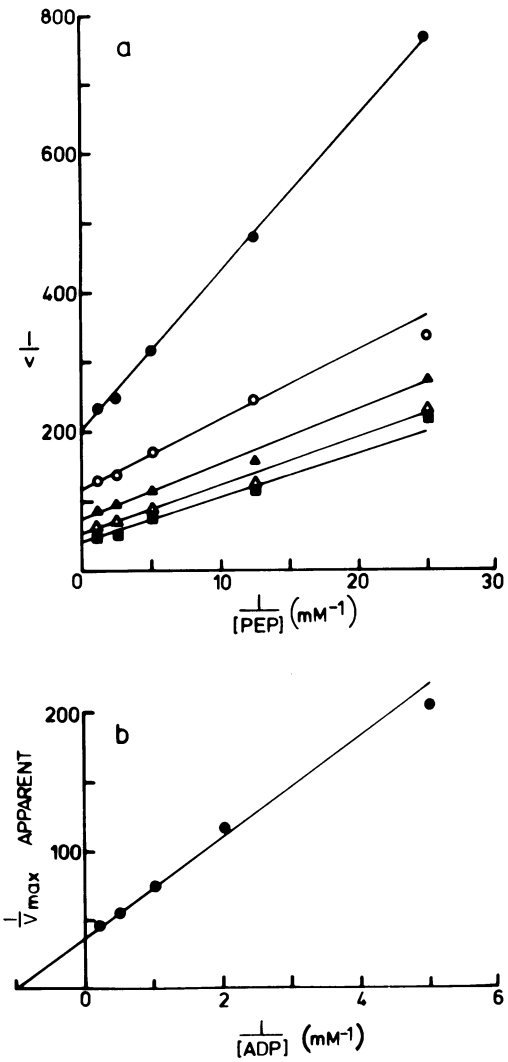

FIG. 4. *K_m value for ADP for S. lactis pyruvate kinase. (a) Relationship between reaction velocity (v, units per milliliter) and PEP concentration with varying concentrations of ADP. The standard assay was used except that the PEP concentration was varied as indicated, and ADP was present at the following concentrations: 0.2 mM, ●; 0.5 mM, ○; 1 mM, ▲; 2 mM, △; 5 mM, ■. The reaction was initiated by adding 0.021 U of pyruvate kinase per ml (0.29 μg of protein per ml). (b) Double-reciprocal plot of apparent V_{max} (units per milliliter), obtained from data in plot (a) versus ADP concentration.*

The glycolytic intermediates PEP and FDP activated the enzyme, whereas P_i and SO_4^{2-} were inhibitory. The mechanisms of P_i and SO_4^{2-} inhibition remain unclear. Inhibition by P_i has also been reported for pyruvate kinase from *B. licheniformis* (21). The inhibitory effect of SO_4^{2-} has not been previously reported for this enzyme from bacteria and should be considered when carrying out pyruvate kinase assays with $(NH_4)_2SO_4$ suspensions of LDH.

Both PEP and ADP showed positive cooperativity, suggesting that the binding of substrate to the free enzyme is facilitated as other substrate molecules bind to the enzyme (8). At low PEP concentrations the glycolytic intermediate FDP markedly increased the activity of pyru-

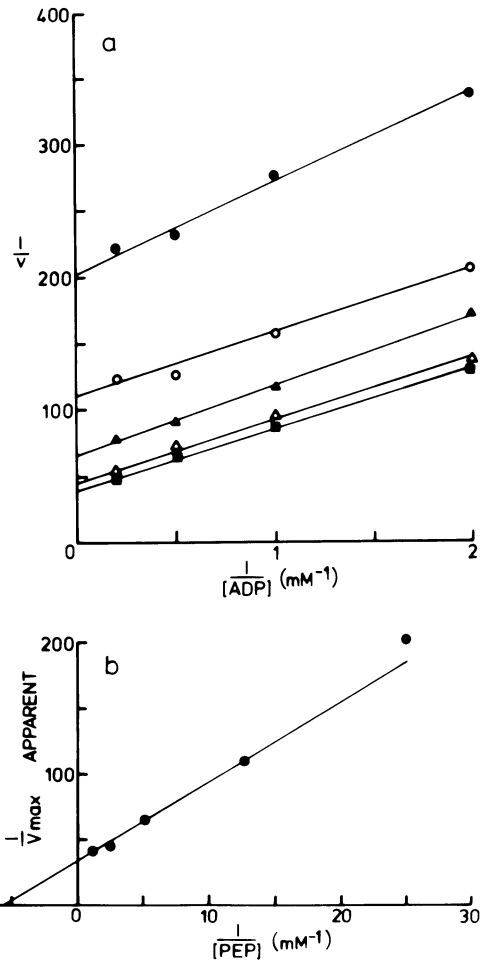

FIG. 5. *K_m value for PEP for S. lactis pyruvate kinase. (a) Relationship between reaction velocity (v, units per milliliter) and ADP concentration with varying concentrations of PEP. The standard assay was used except that the ADP concentration was varied as indicated, and PEP was present at the following concentrations: 0.04 mM, ●; 0.08 mM, ○; 0.2 mM, ▲; 0.4 mM, △; 0.8 mM, ■. The reaction was initiated by adding 0.021 U of pyruvate kinase per ml (0.29 μg of protein per ml). (b) Double-reciprocal plot of apparent V_{max} (units per milliliter), obtained from data in plot (a) versus PEP concentrations.*

TABLE 1. *Intracellular concentration of metabolites in exponentially growing S. lactis ML₃ compared to the concentration giving one-half maximal velocity in vitro*

Metabolite	Intracellular concn[a] (mM)	Concn for ½ V_{max} (mM)
Phosphoenolpyruvate	0.76 ± 0.15	0.17[b]
Adenosine diphosphate	2.4 ± 0.3	1.0[b]
Fructose-1,6-diphosphate...	18.3 ± 1.8	0.07[c]

[a] Mean with standard deviation for four extracts prepared from two different cultures by procedures described in the text.

[b] K_m value in the presence of 2 mM FDP.

[c] Value obtained in the presence of 2 mM PEP and 5 mM ADP.

vate kinase. FDP decreased the K_m values for both PEP and ADP, indicating that FDP increases the affinity of the enzyme for both substrates. In view of the sigmoidal PEP saturation curve and the marked activation by FDP, the pyruvate kinase from *S. lactis* can be considered as an allosteric protein. Activation of pyruvate kinase from *S. lactis* by FDP has not been previously reported, although FDP activation of LDH from *S. cremoris* is well documented (6). The K_m values for PEP and ADP in the presence of 2 mM FDP were 0.17 and 1 mM, respectively. Similar values were obtained with pyruvate kinase from other organisms (3, 7, 14, 16, 21). PEP is required for the transport of carbohydrates into *S. lactis* by the PEP-phosphotransferase system (11, 12). The regulation of pyruvate kinase by FDP could control the intracellular concentration of PEP, indirectly controlling the rate of sugar transport into the cell. This control may be an important mechanism for regulation of the glycolytic pathway in lactic streptococci.

No evidence was obtained for the activation of *S. lactis* pyruvate kinase by AMP, though such activation has been reported for this enzyme from *Brevibacterium flavum* (16) and *B. licheniformis* (21). Regulation of pyruvate kinase in these organisms is by AMP and ADP activation and ATP inhibition. At low concentrations of FDP (less than 0.8 mM), pyruvate kinase from *S. lactis* was inhibited by ATP. However, this effect is unlikely to apply in exponentially growing cells where the FDP concentration is 18 mM. Activation by PEP and ADP has been found with pyruvate kinase from other organisms (3, 5, 21). Pyruvate kinase from *B. subtilis* is not activated by either AMP or FDP (3). *E. coli* has two forms of pyruvate kinase, one of which is activated by AMP and the other by FDP (7, 14).

The intracellular concentrations of PEP, ADP, and FDP in *S. lactis* ML_3 are considerably higher than the concentrations required for one-half maximal velocity in vitro, suggesting that the pyruvate kinase of this organism approaches maximal activity in exponentially growing cells. The intracellular concentrations of PEP and FDP in *S. lactis* were approximately ten times (PEP) and six times (FDP) greater than in growing cells of both *E. coli* (9) and yeast (4), whereas the intracellular concentration of ADP in *S. lactis* was approximately three times the concentration found in *E. coli* (9). The dangers of extrapolating from observations in vitro involving dilute enzyme systems, to conditions in vivo, have been pointed out by Srere (17). However, Kornberg and Malcovati (7) have found that the kinetic properties of pyruvate kinase in "permeabilized" *E. coli* were similar to those observed in vitro, and it seems reasonable to assume that the regulatory properties observed for pyruvate kinase from *S. lactis* are indicative of properties in vivo.

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